Nesiotobacter exalbescens gen. nov., sp. nov., a moderately thermophilic alphaproteobacterium from an Hawaiian hypersaline lake

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A Gram-negative bacterium, designated LA33B^T, was isolated from water collected from a hypersaline lake on uninhabited Laysan Atoll in the Northwestern Hawaiian Islands. Cells of strain LA33B^T are motile, straight rods that grow between 4 and 45 °C and in media containing 1–17·5 % (w/v) NaCl. The strain oxidizes carbohydrates, nucleosides, amino acids and organic acids presented as sole carbon sources and constitutive lipolytic and proteolytic enzymes are expressed. Over 75 % of the fatty acid pool is *cis*-11-octadecenoic acid (18 : 1ω7*c*). Comparative sequence analysis of the 16S rRNA gene indicates that the strain forms a new lineage in the α-2 subclass of the *Proteobacteria*, with the closest recognized strains being *Stappia aggregata* NCIMB 2208^T and *Roseibium denhamense* JCM 10543^T, with which it shares 94–95 % sequence similarity. Strain LA33B^T differs phenotypically from extant *Stappia* and *Roseibium* species, however, in that it is a moderate thermophile, it requires NaCl and tolerates higher NaCl concentrations and it does not express β-galactosidase or oxidize glycerol. On the basis of genotypic data and phenotypic characteristics, we propose that strain LA33B^T does not belong to the genera *Stappia* or *Roseibium* and that it represents the type species of a new genus, *Nesiotobacter*. Strain LA33B^T (= ATCC BAA-994^T = CIP 108449^T) is proposed as the type strain of the type species of this genus, with the name *Nesiotobacter exalbescens* gen. nov., sp. nov.

Water collected from the hypersaline lagoon at the centre of uninhabited Laysan (25° 46′ N 171° 44′ W) in the Northwestern Hawaiian Islands was spread on ASP medium, a minimal medium containing (l^{-1}) 1 g aspartic acid (monosodium salt), 10 g glycerol, 1 g K_2HPO_4, 15 g agar, 40 g NaCl and 1 ml SL-8 micronutrient solution (Atlas, 1997). After incubation in darkness (25 °C, 7 days), representative colonies were transferred to marine agar 2216E (MA; Difco) and incubated at 30 °C (Donachie *et al.*, 2004a). Strain LA33B^T grew as off-white, 1–2 mm, translucent colonies. Working cultures were maintained on MA. Stock cultures were stored at −80 °C in marine broth (MB; Difco) and glycerol (30 % w/v).

Tolerance of NaCl was tested on tryptic soy agar (TSA; BBL) containing 0·5–20 % (w/v) NaCl at 30 °C for 10 days and in 50 % MB with 1–20 % NaCl (w/v); 50 % MB is half-strength MB prepared with distilled water and supplemented with NaCl in a range from 1 to 20 % (w/v). Growth was followed for 131 h by measuring turbidity at 600 nm in a spectrophotometer (DU 650; Beckman). The temperature range for growth was tested on MA from 4 to 58 °C. Motility was determined in a hanging drop under a 100× objective with oil immersion. Microaerophilic growth on MA was checked in the GasPak Pouch system (BBL) with oxygen and carbon dioxide concentrations of <2 and >4 %, respectively. Cells grown for 72 h in MB were prepared for scanning electron microscopy (Donachie *et al.*, 2002).

Single colonies of LA33B^T on MA were tested for catalase and cytochrome oxidase *c* activities with 3 % (v/v) hydrogen peroxide (Sigma) and 1 % tetramethyl-*p*-phenylenediamine on paper discs (BBL), respectively. Nitrate reduction was determined in nitrate broth (Difco) containing NaCl to 7·5 % (w/v) with standard reagents added after 48 h at 30 °C and in API 20NE tests supplemented with 2 % (w/v) NaCl (bioMérieux). Amylase activity, constitutive enzyme activities (API ZYM) and oxidation of single carbon sources (Biolog GN) were tested as described previously (Donachie *et al.*, 2004b). Growth on, and acidification of, single carbohydrates were tested in API 50CHB/E medium (bioMérieux), with the salinity of the medium adjusted to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Nesiotobacter exalbescens* LA33B^T is AF513441.

A scanning electron micrograph of *Nesiotobacter exalbescens* cells, growth curves in relation to salinity and a fatty acid profile of whole cells are available as supplementary material in IJSEM Online.

2 % (w/v) by the addition of sterile 20 % (w/v) NaCl. Absorption spectra of cells grown aerobically on MA at 30 °C for 48 h and suspended in 60 % sucrose (in distilled water) and of the same cells extracted with methanol (overnight, 4 °C) were determined between 400 and 900 nm in a spectrophotometer (DU 650; Beckman) (Nishimura *et al.*, 1994; Uchino *et al.*, 1998). Manganese oxidation was tested on medium K (Rosson & Nealson, 1982). Fatty acids in cells grown on MA at 30 °C were determined commercially (Sasser, 1997).

Genomic DNA was extracted from a 72 h culture in MB (Marmur, 1961; Donachie *et al.*, 2004a) and a fragment of the 16S rRNA gene was amplified by PCR with *Pfu* DNA polymerase and primers 27F and 1492R (Lane, 1991; Donachie *et al.*, 2004b). A consensus of sequences, manually edited and assembled in SEQMAN (Lasergene), was compared with those in the public domain through a BLAST*n* search (Altschul *et al.*, 1997). The phylogenetic relationship of LA33B^T with type strains from related genera was viewed in a phylogenetic tree based on a neighbour-joining alignment of their 16S rRNA gene sequences (Saitou & Nei, 1987) and corrected for multiple substitutions. DNA G+C content was determined after Mesbah *et al.* (1989). Genomic DNA was hybridized with that from *Stappia aggregata* NCIMB 2208^T and *Roseibium denhamense* JCM 10543^T (Donachie *et al.*, 2004b).

Morphological and physiological characteristics of strain LA33B^T are given in the species description and Table 1. Additionally, the intensity of a pink pigment secreted into the medium fades with time. Cells are single, straight rods (see Supplementary Fig. S1 in IJSEM Online). Optimal salinity for growth in 50 % MB is 1–2 % (w/v) NaCl, but growth occurs at up to 17·5 % (w/v) NaCl after ~80 h (see Supplementary Fig. S2 in IJSEM Online). In this respect, strain LA33B^T is well suited to survival in the hypersaline lagoon from which it was isolated, where salinity has been reported to vary between 40 and 150 ‰ (Warner, 1963; Maciolek, 1982; Donachie *et al.*, 2004a). Growth also occurs on MA at 45 °C. Colonies develop slowly in a CO_2-enriched atmosphere. In nitrate reduction broth, nitrate is not reduced to nitrogen in the absence of NaCl or in the presence of 3·2 % (w/v) NaCl, but reduction does proceed in the presence of 7·5 % (w/v) NaCl. In API 20NE, however, LA33B^T reduced nitrate to nitrogen in the presence of 2 % (w/v) NaCl. Amylase was not detected.

Over 99 % of the fatty acids in whole cells were named, including *cis*-11-octadecenoic acid (18 : 1ω7*c*, 77·72 %), hexadecanoic acid (16 : 0, 5·29 %) and octadecanoic acid (18 : 0, 5·22 %). Several other fatty acids each comprised less than 5 % of the total (see Supplementary Table S1 in IJSEM Online). Diverse carbon sources are oxidized in the Biolog GN, namely dextrin, glycogen, sucrose, D-fructose, maltose, D-mannose, α-D-glucose, trehalose, turanose, *N*-acetylglucosamine, glucuronamide, α-ketoglutaric acid, β-hydroxybutyric acid, acetic acid, DL-lactic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, L-glutamic acid, L-pyroglutamic acid, *p*-hydroxyphenylacetic acid, D-gluconic acid, propionic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, inosine, thymidine, uridine, L-alaninamide, L-phenylalanine, L-proline, L-serine, Tween 40 and Tween 80. Oxidation of D-psicose, D-serine and methyl β-D-glucoside is weak. Substrate use that differentiates strain LA33B^T from closely related genera is shown in Table 1. Acid production from carbohydrates in API 50CHB/E is given in the species description; acid production from sucrose, maltose and D-xylose is weak. The strain also grows weakly in API 50CHB/E on D-mannose and gluconate, but these substrates are also oxidized, along with ribose, D-glucose, D-fructose, methyl α-D-glucoside and *N*-acetylglucosamine. Constitutive enzyme activities detected in API ZYM are given in the species description. No absorption peaks were determined in whole cells in sucrose or in methanol extracts, suggesting that photopigments are absent. Brown colonies on medium K are indicative of manganese oxidation.

The 16S rRNA gene sequence of strain LA33B^T falls within the α-2 subclass of the *Proteobacteria*, with the closest described neighbours being *S. aggregata* NCIMB 2208^T and *R. denhamense* JCM 10543^T, which share 94·2 % sequence similarity over 1384 nucleotides and 95 % over 1363 nucleotides, respectively (Fig. 1). Hybridization of strain LA33B^T DNA with that of *S. aggregata* NCIMB 2208^T and *R. denhamense* JCM 10543^T showed 15 and 14 % reassociation, respectively. Strain LA33B^T cannot be considered to belong to either of these species when the recommendation of the ad hoc committee is considered (Wayne *et al.*, 1987). Although the DNA G+C content and major fatty acid of *R. denhamense* JCM 10543^T and strain LA33B^T are similar (Suzuki *et al.*, 2000), these strains can be differentiated based on their degree of DNA–DNA reassociation, their maximum growth temperatures and their tolerance of NaCl (Table 1). Neither type strain of the two *Roseibium* species grows in the presence of 13·5 % (w/v) NaCl (Suzuki *et al.*, 2000). Strain LA33B^T can also be distinguished from *S. aggregata* by the degree of DNA–DNA reassociation, different pigmentation and the fact that *S. aggregata* does not grow at 45 °C. Phenotypic characteristics of particular value in differentiating LA33B^T from members of these and related genera are growth at elevated temperatures, response to salinity, oxidation of substrates in Biolog GN, reduction of nitrate to nitrogen, absence of β-galactosidase (ONPG) and inability to oxidize D-galactose, gentiobiose, D-mannitol or glycerol (Table 1). We propose that LA33B^T is the type strain of the type species of a new genus, *Nesiotobacter* gen. nov., and propose the name *Nesiotobacter exalbescens* gen. nov., sp. nov.

Description of *Nesiotobacter* gen. nov.

Nesiotobacter (Ne.si′o.to.bac′ter. Gr. adj. *nesiotes* of an island, insular; N.L. masc. n. *bacter* from Gr. n. *bakterion* rod; N.L. masc. n. *Nesiotobacter* rod from an island, in this case Laysan).

Table 1. Phenotypic data that differentiate *Nesiotobacter exalbescens* LA33B^T from phylogenetically related species

Species: 1, *Nesiotobacter exalbescens* LA33B^T; 2, *Roseibium denhamense* JCM 10543^T (data from Nishimura *et al.*, 1994; Suzuki *et al.*, 2000; this study); 3, *Roseibium hamelinense* CIP 107048^T (Nishimura *et al.*, 1994; Suzuki *et al.*, 2000; this study); 4, *Stappia stellulata* CIP 105977^T (Uchino *et al.*, 1998; this study); 5, *Stappia aggregata* NCIMB 2208^T (Rüger & Höfle, 1992; Uchino *et al.*, 1998; this study); 6, *Rhodobium orientis* JCM 9337^T (Hiraishi *et al.*, 1995); 7, *Phyllobacterium myrsinacearum* ATCC 43590^T (Lambert *et al.*, 1990; Swings *et al.*, 1992; de Lajudie *et al.*, 1994; Mergaert *et al.*, 2002); 8, *Defluvibacter lusatiensis* DSM 11099^T (Fritsche *et al.*, 1999); 9, *Aquamicrobium defluvii* DSM 11603^T (Bambauer *et al.*, 1998). All strains are motile. Results refer to the type strain only in most cases. Characteristics of particular value in differentiating strain LA33B^T from members of related genera are no growth in the absence of NaCl, but growth in the presence of 10 or 13·5% (w/v) NaCl, growth at 45 °C, reduction of NO_2^- to N_2, inability to hydrolyse urea and lack of indole production in API 20NE and 20E tests and oxidation of Tween 80, but not of L-arabinose, D-cellobiose, D-galactose, gentiobiose, D-mannitol or glycerol in Biolog GN tests. Empty cells indicate that no data are available. (+), Weak to no growth; W, weak; V, variable (includes data reported in Rüger & Höfle, 1992; Uchino *et al.*, 1998; Suzuki *et al.*, 2000).

Characteristic	1	2	3	4	5	6	7	8	9
Colour*	B to Wh	P	P	B	C, B, Y	P, R, C	C to B	WhG	W
Cell size (μm)	0·4–0·6 × 1·2–4	0·5–0·8 × 1–4	0·5–0·8 × 1–4	0·6–1 × 2–4	0·6–1·2 × 1–4	0·7–0·9 × 1·5–3·2	0·4–0·8 × 0·4–1·4	0·6–0·8 × 1·4–3	0·5–0·8 × 1·5–2·5
Growth in NaCl (% w/v):									
0	−	−	+	(+)	(+)				
1	+	+	+	+	+	−			+
5	+	+	+	+	+	+			−
10	+	+	+	−	−	−			−
13·5	+	−	−	−	−	−			−
Growth at:									
35 °C	+	+	+	+	+		+	+	+
40 °C	+	−	−	−	−		−	+	+
45 °C	+	−	−	−	−		−	−	−
Anaerobic growth	+	−	−	−	(+)				+
Bacteriochlorophyll *a*	−	+				+			
NO_3^- to NO_2^-†	+	+	+	−	V	+	+	−	+
NO_2^- to N_2†	+	−	−	+	+	+	+	−	−
Hydrolysis of:									
Gelatin†	+	V	V	−	−				
Starch	−	−	−	−	−				
Tween 80	+	V	V				−		
Urea†	−	+	−	−	−				
ONPG†	−	+	+	+	+				
Indole†	−	+	+	−	−				
VP test†	−	−	−	−	−				
Carbon substrate tests (Biolog GN system):									
Acid from D-glucose‡	+	+	+	+	+/−		−		W
Adonitol	−	−	−	+	−		+	+	
D-Arabitol	−	−	−	−	−		+	−	
L-Arabinose	−	−	−	+	+	−	+/−	−	
D-Cellobiose	−	+	−	+	+		+	−	
D-Galactose	−	+	+	+	+	+	+	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
Gentiobiose	−	+	+	+	+		+	−	
Glycerol	−	+	+	+	+	−	+	+	
Maltose	+	+	+	+	+		+	−	−
D-Mannitol	−	+	+	+	+	+	+	−	+
D-Trehalose	+	+	−	+	V		+	−	
Turanose	+	+	+	+	+		+	−	
Tween 80	+	−	+	+	+		−		
Xylitol	−	−	−	+	−		+	−	
DNA G+C content (mol%)	61	60·3	61	59	59	65·3	60	61·4	61·7

*B, Beige; C, colourless; G, grey; P, pink; R, red; Wh, white; Y, yellowish.
†API 20NE and API 20E strips supplemented with 2% NaCl. API 20E tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase were negative for strains 1–5.
‡Leifson's O/F medium (for strains 1–5).

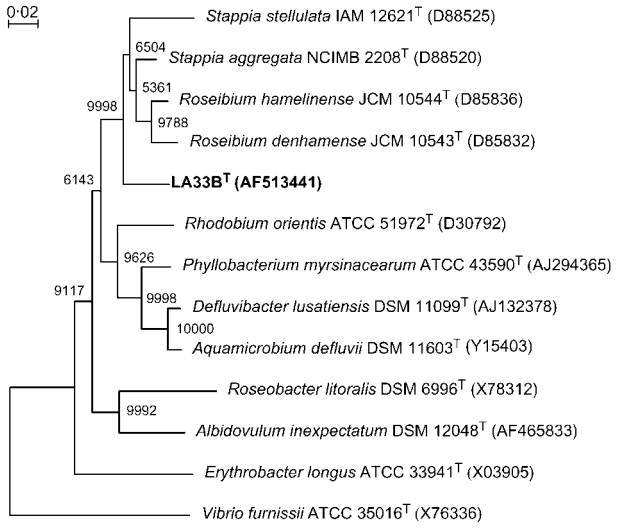

Fig. 1. Phylogenetic tree showing the relationship of *Nesiotobacter exalbescens* LA33B^T to representatives of the *Alphaproteobacteria*, on the basis of 1363 nucleotides from the 16S rRNA gene sequence. Sequences were aligned in CLUSTAL_X. The gammaproteobacterium *Vibrio furnissii* ATCC 35016^T was used as the outgroup. Bootstrap values for 10 000 replicates are shown. The tree was rendered in TREEVIEW (Page, 1996). Bar, 0·02 nucleotide substitutions per site.

Cells are motile, Gram-negative rods that are catalase- and oxidase-positive. Growth occurs in the presence of 13·5 % (w/v) NaCl. Bacteriochlorophyll *a* is absent. Nitrate is reduced to nitrogen. The major fatty acid in cells grown at 30 °C is *cis*-11-octadecenoic acid (18 : 1ω7*c*). Comparative analysis of the nucleotide sequence of the 16S rRNA gene sequence indicates that the genus belongs in the α-2 subclass of the *Proteobacteria*. The type species of the genus is *Nesiotobacter exalbescens*.

Description of *Nesiotobacter exalbescens* sp. nov.

Nesiotobacter exalbescens [ex.al.bes′cens. L. part. adj. *exalbescens* (from L. v. *exalbesco*) becoming white, growing white, referring to the fading colour of maturing colonies.]

Exhibits the following properties in addition to those given in the genus description. Circular colonies, beige, becoming off-white, translucent, flat to slightly raised, entire, smooth, glistening, 2–3 mm in diameter after 24 h at 30 °C on MA. A pink hue that forms under a fresh culture on MA 2216E fades within 72 h. Cells are 0·4–0·6 μm wide by 1·2–4 μm long. Grows on TSA and in half-strength MB containing 0·5–13·5 % and 1–17·5 % (w/v) NaCl, respectively. Grows at 45 °C, but not at 50 °C. Nitrate is reduced to nitrogen in the presence of NaCl. β-Galactosidase is absent. Acid is produced from ribose, D-mannose, gluconate, D-fructose, D-glucose, methyl α-D-glucoside and *N*-acetylglucosamine in API 50CHB/E. Alkaline phosphatase, esterase (C_4),

esterase lipase (C_8), leucine and cystine arylamidases and trypsin are expressed in API ZYM. The DNA G+C content is 61 mol%.

The type strain, LA33B^T (=ATCC BAA-994^T=CIP 108449^T), was isolated from a hypersaline lagoon on Laysan Atoll in the Northwestern Hawaiian Islands.

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